



Extracellular vesicle associated long non-coding RNAs functionally enhance cell viability

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ABSTRACT

Cells communicate with one another to create microenvironments and share resources. One avenue by which cells communicate is through the action of exosomes. Exosomes are extracellular vesicles that are released by one cell and taken up by neighbouring cells. But how exosomes instigate communication between cells has remained largely unknown. We present evidence here that particular long non-coding RNA molecules are preferentially packaged into exosomes. We also find that a specific class of these exosome associated non-coding RNAs functionally modulate cell viability by direct interactions with α -lactate dehydrogenase B (LDHB), high-mobility group protein 17 (HMG-17), and CSF2RB, proteins involved in metabolism, nucleosomal architecture and cell signalling respectively. Knowledge of this endogenous cell to cell pathway, those proteins interacting with exosome associated non-coding transcripts and their interacting domains, could lead to a better understanding of not only cell to cell interactions but also the development of exosome targeted approaches in patient specific cell-based therapies.

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1. Introduction

Exosomes are 40–100 nm extracellular vesicles secreted by mammalian cells, the result of multivesicular endosome fusion to the cells plasma membrane. These extracellular particles are involved in intercellular communication by acting as transport vehicles for proteins and RNA, both mRNA and non-coding RNAs [40]. Small non-coding RNAs, such as miRNAs, as well as long non-coding RNAs (lncRNAs) have also been observed packaged into exosomes [17] and found to act as messengers in cell-to-cell communication [39]. Non-coding RNAs exhibit a plethora of functions, ranging from chromatin and enhancer modifiers to scaffolding and transcript sponge functions [22]. Some lncRNAs such as MALAT1 and NEAT1 have also been found to function in cancer cell signalling [2,3,5–8,18,20,28], with MALAT1 being previously observed to be packaged into exosomes [5].

One possible mode of action for these particular lncRNAs might be to act distally in the creation of a pre-metastatic niche for metastatic cells [1,10]. We set out to explore this notion and find a set of exosome-associated lncRNAs that functionally modulate cell viability by interactions with several proteins involved in cellular metabolism and signalling. Previous studies have identified that RNA content can be dependent on the cell type from which it originated from. This has led to an increase in the investigation of exosomal RNAs as possible biomarkers for a variety of cancers [26,27,33]. It has also been observed that Viruses are capable of affecting RNA content within exosomes, which can repress the target genes of the virus [4,29]. In light of these discoveries we set out to determine whether a cervical cancer affected by the HPV virus (in this case Hela) affect the RNA content in the exosome when compared to a cervical cell line without HPV (C33A).

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2. Materials and methods

2.1. Cell culture

Hela and C33A cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution and 2 mM L-glutamine. The cells were grown in T-175 flasks at 37 °C and 5% CO₂. Cultures were passaged when they were ~90% confluent. The media was removed from the cells and they were washed with 8 mL of 1X PBS. Trypan blue exclusion was used to assess cell viability and estimate cell numbers.

2.2. Calcium phosphate transfection of Hela cells

Hela cells were transfected with 5-Bromouridine 5'-Triphosphate (BrUTP) using the calcium phosphate transfection method [15]. When cells take up the BrUTP, it is utilised in transcription and incorporated into RNA transcripts causing them to be labelled. The BrUTP-labelled transcripts are packaged into exosomes, which are isolated from the cell culture media, described below. One day prior to transfection, 100 mm cell culture plates were seeded with approximately 2 million Hela cells and the volume of each dish was made up to 10 mL with fresh media. For each plate, a transfection mix was made by adding 500 µL of 1X HBS and 0.5 µL of 10 mM BrUTP to a sterile microcentrifuge tube and vortexing to mix. To each transfection mix 30 µL of 2.5 M calcium chloride was added. The tubes were mixed by vortexing and left to sit at room temperature for 20 min. The calcium phosphate transfection mix was added to each plate drop-wise and mixed by rocking. The final BrUTP concentration in each plate was 0.5 µM. Exosomes from the cell culture media were collected over a 3 day period post-transfection.

2.3. Exosome isolation

Exosomes were isolated from conditioned media as they are secreted by cells into the culture media. During cell passaging, the conditioned media was collected and centrifuged at 700 ×g for 5 min to remove any live cells. The conditioned media was stored at –20 °C. To isolate exosomes from the conditioned media, ultracentrifugation on a 30% sucrose density cushion was used. The conditioned media was centrifuged at 10,000 ×g for 30 min with a Hitachi P28S rotor to remove any dead cells or cellular debris. The supernatants were then transferred to clean 40 PA tubes and centrifuged at 100,000 ×g for 90 min in the P28S rotor to pellet the exosomes and contaminating proteins. The supernatant was discarded and the pellet was resuspended with 1 mL of 1X PBS. The resuspended exosomes were pooled together and the total volume was made up to 9 mL with 1X PBS. In a 13 PA tube, 1 mL of the 30% sucrose density cushion (30 g sucrose, 2.4 g Tris base, 100 mL deuterium oxide, pH 7.4) was added and the resuspended exosome solution was placed above the sucrose cushion. Great care was taken not to disturb the interface between the two solutions. Using a Hitachi P40S rotor, the sucrose cushion and exosome solution was centrifuged at 100,000 ×g for 90 min. During this ultracentrifugation step contaminating proteins are pelleted while the exosomes float in the sucrose cushion. The sucrose cushion was transferred to a clean 13 PA; 9 mL of 1X PBS was added and the exosomes were washed by centrifuging at 100,000 ×g for 90 min. The supernatant was removed and final exosome pellet was resuspended in 100 µL of 1X PBS. All centrifugation steps were performed at 4 °C using a Hitachi HiMac CP100WX ultracentrifuge. A Bradford protein assay (Bio-Rad) was performed to estimate the exosome concentration.

2.4. Western blot of exosomal markers

Western blots of exosomal markers CD9, CD63 and flotillin-1 were performed to confirm the isolation of exosomes. The cellular protein calnexin was also analysed as a control, as it is not present in exosomes. The anti-CD9 and anti-CD63 antibodies required non-reducing conditions while reducing conditions were required for the anti-flotillin-1 and anti-calnexin antibodies required. Hela exosomes (10 µg) and cells (10⁵) were mixed with either non-reducing sample buffer (4X Laemmli sample buffer) or reducing sample buffer (4X Laemmli sample buffer with 10% 2-mercaptoethanol) depending on the condition required by the specific antibody. The samples in the reducing buffer were heated at 95 °C for 10 min. The samples and Precision Plus Protein Kaleidoscope Standard were loaded onto a 4–20% gradient Mini-PROTEAN TGX precast gel. Electrophoresis was performed at 150 V for 45 min in 1X Tris/Glycine/SDS buffer. The proteins in the gel were transferred onto to a PVDF membrane using a Bio-Rad Mini-Trans Blot Electrophoretic Transfer Cell at 90 V for 1 h in 1X Tris/glycine buffer with 20% methanol. The PVDF membranes were blocked overnight at 4 °C in 15 mL of 1X PBS/5% skim milk/0.1% Tween-20 blocking solution. The anti-CD9 (ab2215), anti-CD63 (ab59479) and anti-calnexin (ab21290) primary antibodies were diluted 1:1000 in blocking solution and the anti-flotillin-1 antibody (ab41927) was diluted 1:500. The membranes were incubated with 3 mL of a specific primary antibody solution for 4 h at room temperature on a shaking platform. They were then washed three times for 10 min in 50 mL of 1X PBS/0.1% Tween-20 at room temperature with shaking. The anti-mouse IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad) was diluted 1:3000 times in blocking solution. The membranes were incubated in 3 mL of secondary antibody solution for 1 h at room temperature with shaking before being washed again three times. Bound secondary antibodies were colorimetrically detected using an Opti-4CN Substrate Kit.

2.5. Electron microscopy of exosomes

Electron microscopy of exosomes was performed by Rebekka Williams of the Children's Cancer Institute Australia (as a fee for service) to confirm that exosomes were isolated from conditioned cell culture media. A 10 µL suspension of Hela exosomes was fixed with 2% glutaraldehyde for 30 min. The fixed exosomes (5 µL) were dropped onto 300 mesh carbon coated formvar-copper TEM grids and left to settle for 15 min. Excess sample was removed by wicking with filter paper and the grids were washed with water droplets. The exosomes were negatively stained by placing the grid on a droplet of 2% uranyl acetate for 2 min and any excess was wicked off using filter paper. A Joel JEM-1400 transmission electron microscope was used to image the grids. Negative control grids containing no exosomes (only glutaraldehyde and PBS) were also prepared.

2.6. Sequencing of exosomal RNA

The Ramaciotti Centre for Gene Function Analysis performed library preparation and sequencing of Hela and C33A exosomal RNA on an Illumina HiSeq 2000 as a fee for service. The 100 bp paired end reads generated by RNA sequencing were analysed by first quality control performed using FastQC v0.10.1 available from <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>. Reads were quality filtered, trimmed and adapter sequences were removed using Trimmomatic v0.3.0 [21]. Reads were then aligned to the hg19 *Homo sapiens* genome with tophat2 (v2.0.8b) using the default settings except for the following (–b2-sensitive) [14]. Transcripts were assembled using Cufflinks v2.1.1 [38]. All

transcripts were merged using cuffmerge and FPKM values were determined using cuffdiff.

2.7. Fluorescence-activated cell sorting (FACS)

FACS was used to demonstrate the transfer of RNA from exosomes into recipient cells. This was achieved by exposing C33A cells to HeLa exosomes (20 μ l) containing either normal RNA or BrUTP-labelled RNA for 24 or 48 h. The exosome exposed cells were collected, permeabilised and stained with an anti-BrdU primary antibody (B8434) and an IgG-FITC secondary antibody (F0257) (Sigma–Aldrich). The C33A exosome exposed cells were analysed with a BD Biosciences FACSCanto II flow cytometer at the Biological Resources Imaging Laboratory at UNSW as a fee for service.

2.8. Chromatin immunoprecipitation of biotin linked RNAs

Chromatin immunoprecipitation (ChIP) was performed on T7 expressed biotin dUTP containing lncNRAs (Table S1). The biotin RNAs were generated as described in [13,32]. The Biotin containing RNAs were transfected into HEK293 cells and CHIP carried out 48 h later (as described in [13]). The enrichment of the various RNAs at predicted target loci (Fig. S2) was determined by qPCR with various primers (Table S2).

2.9. Immunoprecipitation and mass spectrometry of RNA associated proteins

Proteins associated with exosomal RNAs were determined by transfection of biotin labelled RNAs (Table S1) into 293HEK cells and immunoprecipitation carried out 48 hrs later from either the cells or exosomes collected from the transfected cells (as described in [9,32]). The resultant elutes were then analysed by mass spectrometry using the fee for service Bioanalytical Mass Spectrometry Facility at UNSW.

3. Results

To explore the notion that particular lncRNAs are preferentially packaged into exosomes we collected extracellular particles containing exosomes from cultures grown in pre-cleared conditions (Fig. 1A–B). Western blot analysis for exosomal markers CD9 and Flotillin-1 contrasted with the cellular protein Calnexin [36] confirmed that the predominant isolate from the extracellular particles utilized here contained known exosomal associated proteins (Fig. 1C). CD9 is a tetraspanin found in cells but also recognized as an exosomal marker as they are found highly enriched on the surface of exosomes and Flotillin-1 is a lipid raft associated protein found in exosomes while Calnexin is an endoplasmic reticulum protein predominately localized in the cell [37]. Collectively, these data suggest that our protocol, based on [31,37] results in the isolation of exosome associated proteins.

Next, we sought to determine the predominant species of lncRNAs associated with these exosomes. HeLa and C33A exosomes were isolated and their RNA content sequenced using Illumina RNA-sequencing. A strong correlation in the RNA content of HeLa and C33A exosomes was observed as determined and those RNA transcripts greater than 4 fragments per kilobase of exon per million fragments mapped (FPKM) plotted against each other (Fig. 1D). The observed R2 value of 0.9584 suggested that the RNA content of HeLa and C33A exosomes is approximately 96% similar (Fig. 1D). Notably, some of the previously observed cancer-exosome associated lncRNAs BCYRN1 [12], MALAT1 [8], GAS5 [16,23,24,34] and NEAT1 [2,7,20,28,35,41], were not observed present above cellular RNAs in the HeLa and C33A exosomes assessed here (Fig. 1E

and Table S3). Interestingly, the top 4 most abundant transcript candidates observed associated with both HeLa and C33A exosomes were un-annotated transcripts emanating from genomic deserts that contained DNase hypersensitive regions of high histone acetylation as well as RNAPII and CTCF binding sites. Mapping to Human Feb. 2009 (GRCh37/hg19) assembly suggested that these transcripts are ribosomal RNAs (Fig. S1A–D). These transcripts were designated Exo1–4 (Figs. 1E, S1A–D and Tables S1 and S3) and were contrasted with the enzymatic RNA, RMRP, a mitochondrial RNA-processing endoribonuclease that is imported into the mitochondria to cleave mitochondrial RNA [11], which was also observed to be lowly associated with the HeLa and C33A exosomes (Fig. 1E, Tables S1 and S2).

Previous studies have demonstrated that lncRNAs can be transmitted from one cell to another by the action of the exosome [40]. To determine to what extent the exosome associated transcripts are transmitted between HeLa and C33A cells, a BrUTP cellular RNA labelling assay was performed. In this assay 5-Bromouridine 5'-Triphosphate UTPs (BrUTP) are transfected into HeLa cells and the resultant cellular exosomes collected 24–48 hrs post-transfection. The exposure of C33A cells to these BrUTP labelled RNA containing exosomes demonstrated that HeLa exosomes can transfect C33A cells and disseminate BrUTP transcripts to ~34% of the treated cells over a 48hr time course (Fig. 2A–C). To determine to what extent these HeLa derived exosomes can affect HEK293 cell function, HEK293 cells were exposed to HeLa cell derived exosomes. Notably, HeLa exosome treatment of HEK293 cells results in significant changes in cell viability, suggesting that these exosomes functionally modulate the recipient target cells (Fig. 2D). This observed effect on cell viability was dosage dependent, suggesting that the lncRNAs exhibit a bona fide and quantitative effect on the recipient cells (Fig. S2). To determine to what extent the candidate non-coding RNAs, Exo1–4 and RMRP are functionally involved in modulating recipient cells, cultures were transfected and the effects on cell viability determined. All of the top candidate exosome associated lncRNAs, Exo1–4 and RMRP functionally enhanced cell viability relative to controls (Fig. 2E). Collectively these observations suggest that exosomes contain a subset of lncRNAs that modulate recipient cell function resulting in enhanced recipient cell viability.

The list of functions of non-coding RNAs continues to expand [22]. To determine how the exosome-associated lncRNAs Exo1–4 and RMRP might function in the cell, biotin-labelled lncRNAs were generated, transfected into cells and the resultant complexes eluted and bound protein partners determined by mass-spectrophotometry analysis (MS)(Fig. 3A). Interestingly, relative to the biotin-GFP control transcripts, Exo1–4 and RMRP precipitated with α -lactate dehydrogenase B (LDHB), high-mobility group protein 17 (HMG-17), and CSF2RB; proteins involved in metabolism, nucleosomal architecture and cell signalling respectively (Fig. 3A). Previous studies have found some lncRNAs can associate with homologous containing loci in the genome. To determine to what extent the exosome-associated lncRNAs Exo1–4 and RMRP might be targeting the genome a chromatin immunoprecipitation analysis was carried out with the biotin-labelled lncRNAs. Only RMRP exhibited any binding to homologous containing genomic loci relative to the controls (Fig. 3B). Notably, the RMRP binding locus is on the shoulder of histone acetylation for CCDC107, a gene expressing a coiled-coil containing membrane protein (Fig. 3C) which also contains Rho guanine nucleotide exchange factor 39 (ARHGEF39), a gene known to be involved in cell migration, in overlapping and antisense orientation (Fig. S3). Collectively, these data suggest that the exosome-associated lncRNAs interact with particular cellular proteins to affect cell viability and the majority of

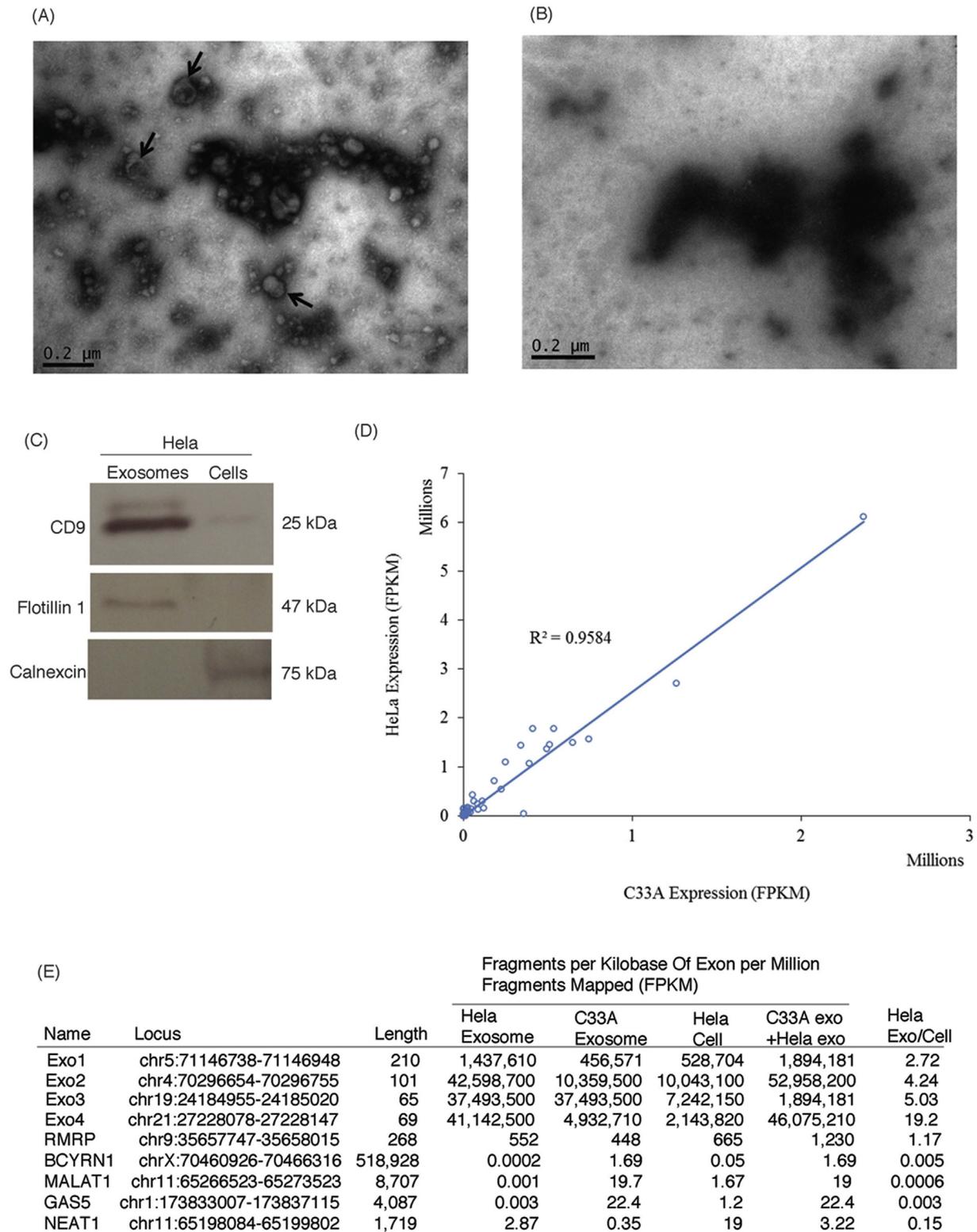


Fig. 1. Analysis of HeLa and C33A associated exosomes – (A–B) Detection of exosomes by electron microscope. (A) a negative control grid with no exosomes and (B) a grid containing HeLa exosomes. The HeLa exosomes are approximately 40 nm–100 nm in size. The three arrows mark exosomes that display the commonly observed cup-shaped morphology. (C) Western blot of exosomal markers. Western blots for the exosomal markers CD9, Flotillin-1 and Calnexin was performed on HeLa exosomal and cellular proteins to confirm that exosomes were isolated from conditioned cell culture media using ultracentrifugation and a 30% sucrose density cushion. (D) Correlation in the RNA content of HeLa and C33A exosomes. HeLa and C33A exosomal RNA transcripts greater than 4 FPKM were plotted against each other and a R2 value of 0.9584 was calculated. (E) Candidate transcripts found in exosomes from both C33A and HeLa cells along with the enrichment of MALAT1, BCYRN1, GAS5 and NEAT1, previously reported in cancer exosomes and the enzymatic RNA RMRP. The top candidate RNAs Exo1–4 and RMRP and were investigated.

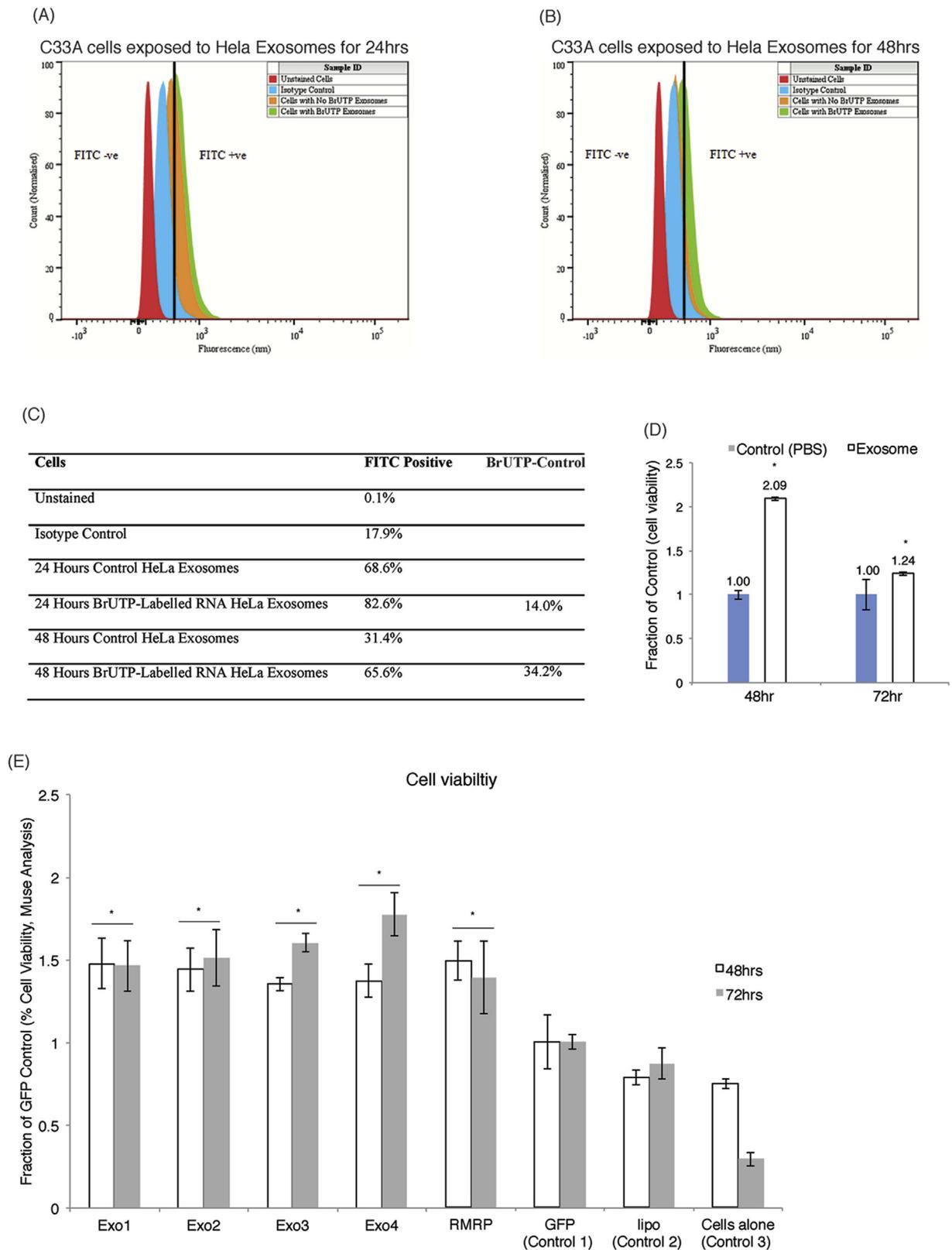


Fig. 2. Exosomal transfer enhances recipient cell viability – (A–C) Exosomal-mediated RNA transfer into recipient cells was determined. C33A cells exposed to HeLa exosomes containing normal RNA or BrUTP-labelled RNA were analysed by FACS to determine if RNA can transfer into cells from exosomes. RNA transfer was determined by FITC fluorescence in cells exposed to exosomes for (A) 24 h or (B) 48 h and (C) the percentage transfer of BrUTP-labelled exosomal RNA determined. (D) Exosomal cell viability of HEK293 cells treated in triplicate with HeLa exosomes or a PBS control. Cell viability was analysed at 48 and 72 h using the Millipore Muse cell analyser. The effects of the top candidate exosome associated RNAs expression on cell viability (E) HEK293 cells were transfected in triplicate to overexpress candidate lncRNAs (Exo1–4, and RMRP) along with a GFP control. Lipofectamine only and untransfected cells served as the controls. Cell viability was measured using the Millipore Muse cell analyser at 48 and 72 h post-transfection. For (D–E) Error bars represent standard error of the mean, *represents $P < 0.05$ relative to GFP Control 1.

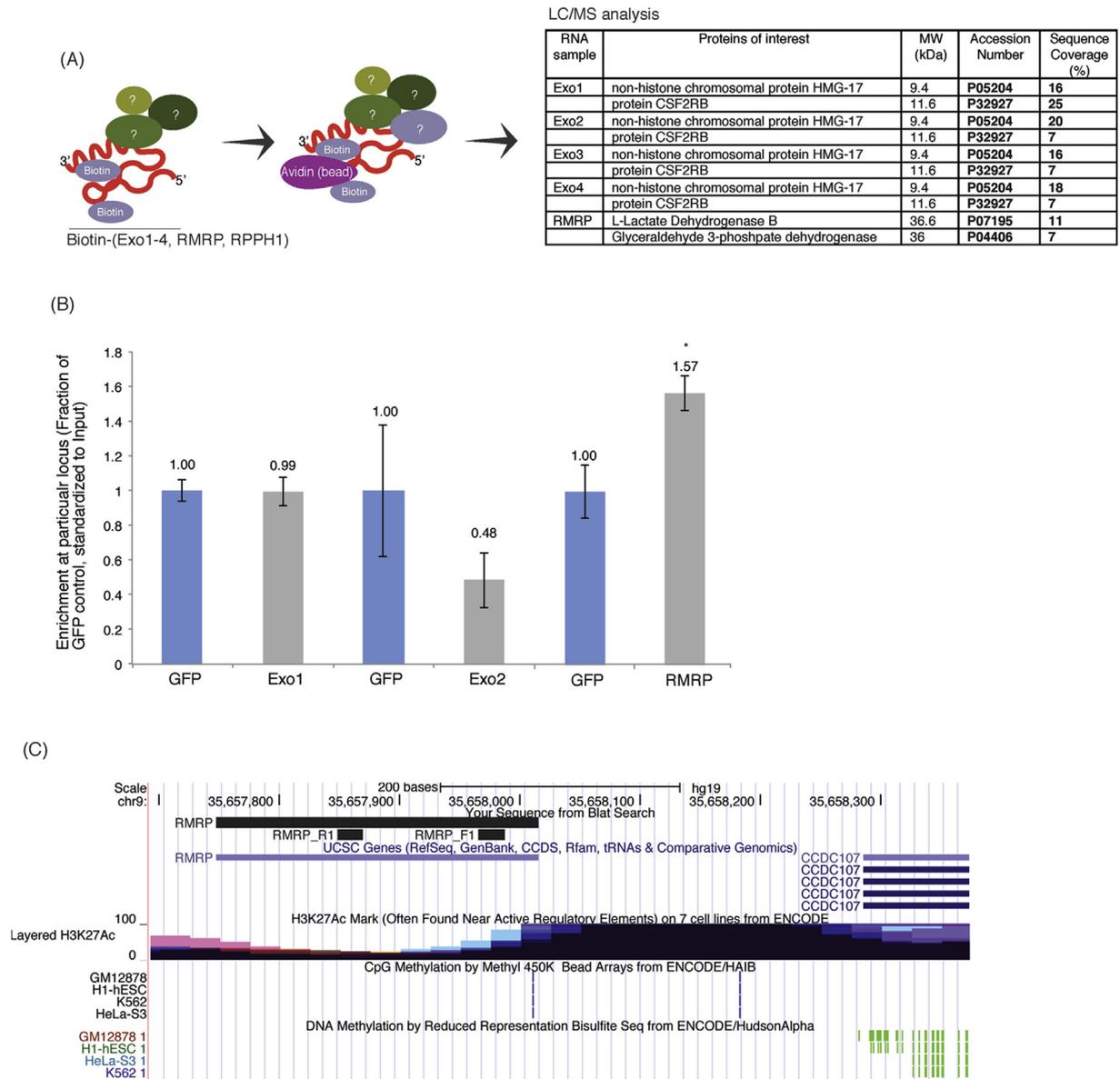


Fig. 3. Mechanism of exosome associated RNA regulation of cell viability and associated proteins – (A) Biotin containing transcripts Exo1-4, and RMRP were transfected into HEK293 cells and elutes from the ChIP sent for mass spec analysis at the UNSW BMFS facility in triplicate. Proteins present in at least two of the samples and not in the GFP controls were flagged as candidates of interest. (B) Relative enrichment of the candidate exosome associated RNAs at homology containing loci. Biotin tagged transcripts (Exo1, Exo2 and RMRP) were transfected into HEK293 cells in triplicate and 48 h later immunoprecipitated with streptavidin beads. Enrichment of each biotin transcript is shown at homology containing loci relative to the GFP control. Error bars represent standard error of the mean, *represents $p < 0.05$. (C) A UCSC snapshot of the RMRP target locus found enriched by CHIP (in B above) is shown with the relative CHIP primer binding sites and associated epigenetic state of the locus.

these interactions, with the exception of RMRP, do not appear to be chromatin based.

The observations that the exosome associated lncRNAs bound to particular proteins (Fig. 3B), suggested that either the candidate lncRNAs bind particular proteins and are packaged into exosomes or functionally interact with the candidate proteins upon being introduced into the cells via exosome uptake in recipient cells. To determine to what extent the exosome associated lncRNAs are interacting with the candidate proteins in either exosomes or recipient cells an immunoprecipitation of the candidate proteins LDH, HGM, CSF and GAPDH (Fig. 3A and B) was carried out in both cells and exosomes and qRT-PCR carried out on the elutes for detection of the candidate transcripts in association with the proteins of interest. Interestingly, the exosome associated lncRNAs only associated with CSF, LDH, and HGM in the context of the recipient

cells (Fig. 4A) and not within the context of the exosome (Fig. 4B). These observations suggest that the exosome-associated lncRNAs interact with particular cellular proteins; CSF, LDH and HGM, upon exosomal entry into the target cells to affect cellular viability.

4. Discussion

To date many studies have been carried out looking at proteins, mRNAs and even miRNAs associated with exosomes (reviewed in [31]). However, little work has been carried out with regards to the association and putative role of lncRNAs in exosome biology. In light of the emerging realizations that lncRNAs play an underappreciated role in controlling chromosomal content, in particular transcriptional and epigenetic states, we sought to determine those lncRNAs associated with both HeLa and C33A cell exosomes. We

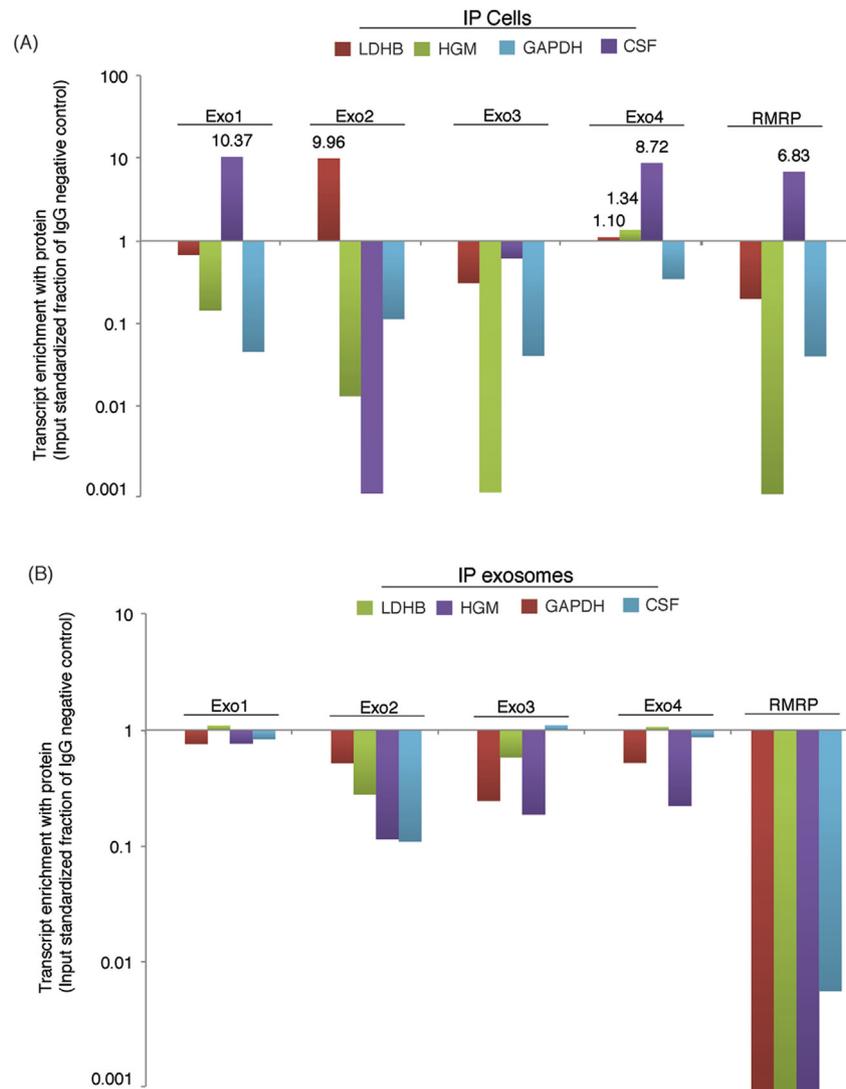


Fig. 4. Enrichment of various exosome-associated lncRNAs with mass spec identified proteins – IP was carried out for the various mass spec identified proteins in triplicate with either (A) cells or (B) exosomes. The resultant elutes were subjected to qRT-PCR and enrichment of each lncRNA with the candidate protein relative to the IgG negative control following input standardization. The averages of triplicate IPs are shown.

find here that exosomes exhibit an abundance of transcripts (Table S3), with the top 4 most abundant exosome associated transcripts in both HeLa and C33A cells being Exo1–4 (Figs. 1, S1 and Tables S1 and S2), that mapped to intergenic gene deserts, several kilobases away from their nearest transcribed coding and/or non-coding neighbour. These transcripts have been considered ribosomal associated transcripts and are found embedded in DNase I hypersensitive clusters with exceedingly high levels of histone H3 at lysine 27 acetylation (H3K27ac), two chromatin marks associated with actively transcribed regions of the genome. Interestingly, we were unable to observe significant enrichment of well-known lncRNAs involved in human cell cancers, BCYRN1, MALAT1, GAS5 and NEAT1, which have been observed previously to be associated with exosomes in human cells and human cell cancers [2,7,8,12,20,28,35,41].

The observations presented here indicate that the top candidate assessed exosome associated lncRNAs Exo1–4 and RMRP, when over-expressed in recipient cells, affect cell viability. Mechanistically, we find that the candidate exosome associated lncRNAs interact directly with several proteins. The exosome associated lncRNA Exo2 appears to interact directly with lactate

dehydrogenase B (LDHB), while Exo4 and RMRP interact with High mobility group protein 17 (HMG-17), and Type I cytokine high affinity receptor for IL-3, IL-5 and CSF (CSF2RB) in cells (Fig. 5). GAPDH is known to bind AU rich transcripts [25] and LDHB is known to bind RNA specifically in the NAD + RNA binding region [30]. Interestingly, LDHB appears to be packaged into exosomes [19], though observations here did not indicate that LDHB was binding directly to any of the exosome associated lncRNAs within the context of the exosome. Regardless, LDHB may be one protein that can bind lncRNAs and recruit them specifically to blebbing exosomes in particular cell types, though the data presented here does not suggest this is the case in HeLa and C33A cells. Notably, the proteins LDHB, GAPDH, CSF and HMG-17 found here to be associated with exosome associated lncRNAs have been reported previously to be involved in cellular metabolism, nucleosomal architecture and cell signalling. It is also noteworthy that RMRP could also have significant impacts on cell viability, when introduced to recipient cells from the context of exosomes, by direct *cis* interactions in the chromatin, possibly affecting CCDC107 and ARHGAP39 expression and cell motility (Fig. S3). The exosome-associated lncRNAs described here don't appear to interact with

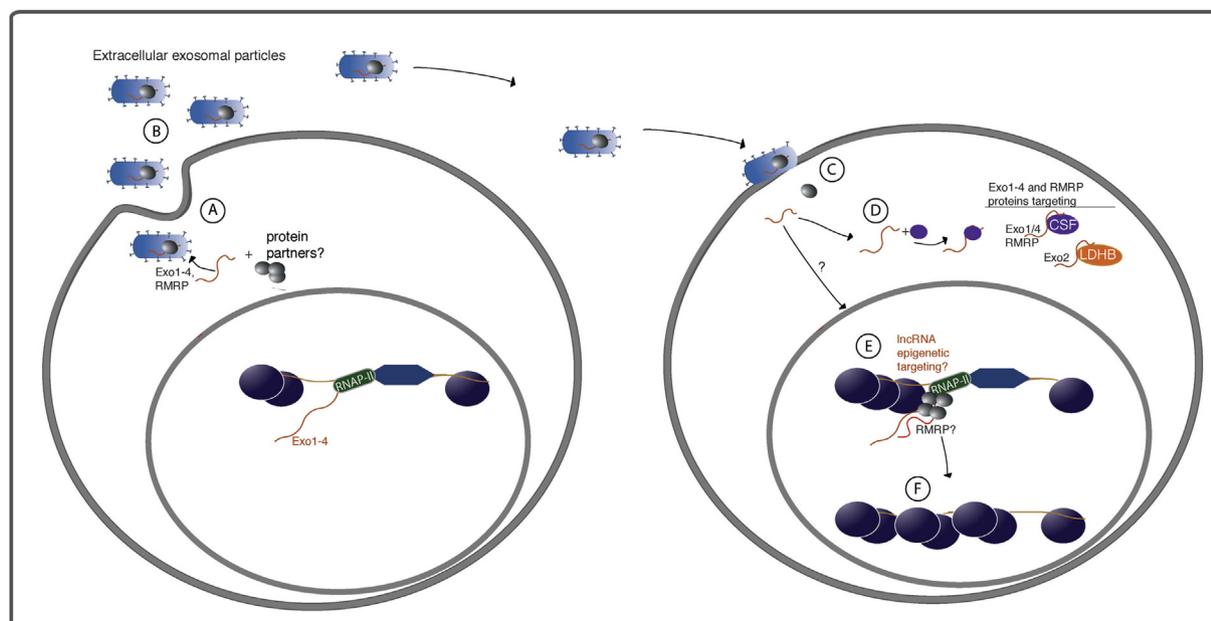


Fig. 5. Model for exosomal mediated spread of lncRNAs. (A) RNAs Exo1–4 and RMRP may interact with host proteins or may be free of interactions and be packaged into exosomes by a specific, yet to be determined set of exosomal directed proteins. (B) The lncRNA containing exosomes are blebbed from the cell and can then (C) interact with recipient target cells. Once inside the recipient cells the lncRNAs can (D) bind proteins, such as Exo1, Exo4 and RMRP binding to CSF or Exo2 binding to LDHB or Exo4 binding HGM-17 (HGM) to affect protein function and cellular states. (E) Some yet to be characterized exosomal-associated lncRNAs such as RMRP may interact with epigenetic regulatory mechanisms to control particular gene expression states by (F) targeting stable epigenetic marks that may lead to targeted heterochromatin.

the cellular proteins in the context of the exosome but rather specifically inside the recipient cells (Fig. 5), suggesting that the regulatory effect these exosome associated transcripts play is upon entry into recipient cells and not within the context of the exosome per se. Collectively, the observations presented here suggest that exosome-associated lncRNAs functionally modulate cellular protein function and expand on the ever-growing functional characteristics of lncRNAs in the human cell. Knowledge of this molecular pathway may prove useful in controlling cellular micro-environments or in developing designer RNA packaged exosomes, an eventuality that could have profound impact on patient specific disease targeted therapeutics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ncrna.2016.06.001>.

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